

REMARKS

Reconsideration and allowance of the subject application is solicited.

In the April 30, 2008 Office Action, claim 1 is rejected under 35 U.S.C. §103(a) as obvious over Blum-Oehler et al (WO 98/44134) in view of Trevors et al. (newly cited). The Examiner cites Blum-Oehler for the disclosure of Nissle 1917 (presumed to be the same as DSM 6601), but acknowledges that this reference does not teach a plasmid-free clone of DSM 6601. However, the Examiner argues that Blum-Oehler teaches that the Nissle 1917 strain has two plasmids (pMut1 and pMut2) that are cryptic and without benefit to the host. The Examiner cites Trevors et al. for teaching methods for removing bacterial plasmids. Combining Blum-Oehler and Trevors, the Examiner has found claim 1 obvious.

Blum-Oehler et al. describes the cryptic plasmids pMUT1 and pMUT2 of E. coli strain Nissle 1917 (syn. DSM 6601). In particular, this reference describes a PCR-based method for the specific detection of E. coli DSM 6601, which can be used to specifically detect E. coli DSM 6601 in the fecal flora of patients in the presence of other E. coli strains. The DNA sequences of the plasmids are described and their use for diagnostic and biotechnological purposes.

However, as noted by the Examiner, Blum-Oehler does not suggest a plasmid-free DSM 6601 strain. The Trevors paper is relied upon for that, and we believe that a fair reading of Trevors would not fill the gap left by Blum-Oehler. Trevors et al. is a review that describes several methods for “curing” bacteria from plasmids; however, none of these methods equate to the actual method we used to construct our plasmid-free strains – which method required significantly more than mere routine procedures. As noted on page 2 of our application:

It turned out in the exhaustive investigations that led to the present invention that plasmid-free clones of strain DSM 6601 cannot be prepared at all with normal genetic engineering methods or can be prepared only with great difficulty so that special paths must be taken in order to generate such clones. Since the wild type of the strain has two plasmids of different sizes in addition to its genomic DNA, the elimination of these plasmids must take place in several steps that take place in part in parallel.

The details of the special paths taken to generate our clones are provided in the text of our application, especially in the Examples. Specific details about the challenges overcome, blind alleys pursued, and the like, are not provided, so at first glance it may appear as if eliminating the pMUT1 and pMUT2 of E. coli strain DSM 6601 was merely routine. This is simply not the case. Please note that our disclosure strongly states that exhaustive investigations were undertaken to overcome the difficulties presented by elimination of the two plasmids of different sizes while preserving the genomic DNA of the E. coli (page 2, et seq). As would be understood by someone having ordinary skill in this art, the general methodologies described by Trevors would not have been useful to remove the two plasmids in E. coli strain DSM 6601. For instance, the inventors discovered that the elimination of the two plasmids must take place in multiple steps, and that some of the steps must be carried out in parallel.

Even assuming arguendo that the desirability of having a plasmid-free E. coli strain DSM 6601 would be known, Trevors' mere description of several different ways to cure bacteria does not equate to successful curing of plasmids pMUT1 and pMUT2 from DSM 6601 such as in claim 1. Blum-Oehler represents the problem in the art solved by our plasmid-free strains. Trevors is a general review article discussing known methods for "curing" bacteria from plasmids – none of which equate with the method our inventors discovered that was ultimately successful to actually eliminate the pMUT1 and pMUT2 plasmids and preserve the genomic DNA in DSM 6601. In other words, as would be understood by someone having ordinary skill in this art, combining Blum-Oehler and Trevors could not have lead to our strain of claim 1. Reconsideration of this rejection is respectfully requested.

Claims 2-6 are rejected under 35 U.S.C. §103(a) as obvious over Uraji et al. (newly cited) in view of Blum-Oehler et al (WO 99/44134) in view of Trevors et al. (newly cited), and in view of Alexeyev et al. (newly cited).

Main claim 2 includes the following steps for preparing a plasmid-free clone for DSM 6601:

- a) introducing a resistance gene into plasmids pMut1 and pMut2,

b) introducing the *sacB* gene into the plasmids [note: this gene involves the conversion of saccharose into a cell-toxic metabolite],

c) introducing the plasmids into the *E. coli* strain DSM 6601 and cultivating the strain under conditions in which the naturally occurring plasmids pMut1 and pMut2 are displaced by the modified plasmids; and

d) cultivating the clones that substantially only permit the growth of *E. coli* population with plasmids that lack the *sacB* gene [note: e.g., culture medium containing saccharose]. It is noted that in our invention the pMut1 may be marked with a tetracycline resistance cassette and pMut2 with a kanamycin resistance cassette.

Regarding the four cited references, the comments above regarding two of them – the Blum-Oehler and Trevors references – are believed to be equally applicable here. That is, Blum-Oehler represents the problem in the art solved by our plasmid-free strains – it presents absolutely no solution to its own problem. Trevors is a general review article discussing known methods for “curing” bacteria from plasmids – none of which equate with the method our inventors discovered that was ultimately successful to actually eliminate the pMUT1 and pMUT2 plasmids and preserve the genomic DNA in DSM 6601.

The other two references, Uraji and Alexeyey, do not make up for the deficiencies of Blum-Oehler and Trevors.

Uraji et al. describes the removal of plasmids in a bacterium via introduction of the *sacB* gene and an kanamycin-resistant cassette. The bacterium already contains a second plasmid without *sacB* gene. The plasmids are removed by cultivation of the bacterium on a culture medium containing saccharose and then on one with kanamycin.

On page 5 of the Office Action, the Examiner has noted that Uraji does not teach the *sacB* gene on the second plasmid (since it is already present in the cell) – however, the Examiner suggested that Uraji does

“teach that *sacB* should be in a plasmid being introduced into the cell that is being cured. Therefore, if two plasmids are being introduced into the cell, it would follow that both plasmids would contain the *sacB* gene in order to cure the cell. In claim 6, the steps are the reverse of the method steps taught by Uraji et al. First, the transformed bacteria are cultivated on plates containing the antibiotic, and then subsequently on plates containing saccharose.” MPEP 2144.04(IV)(C)

is cited.

However, a simple derivation of the method of Uraji (alone or combined with the other cited art) would not produce our modified E. coli DSM 6601. In view of the transformation of the plasmids, simply changing the order of the culture mediums with the order selected in the application would not be obvious or successful. The Uraji article deals with the bacterium agrobacterium and the removal of pTi plasmids. These plasmids are tumor-inducing and can be simply transferred to plants by agrobacterium where in addition to the creation of tumours, these plasmids simultaneously produce nutrients for the bacteria. For these reasons the removal of plasmids from the bacterium is desirable.

Uraji deals with the plant pathogen agrobacterium which is neither genetically, physiologically or microecologically comparable to the probiotic, non-pathogenic E. coli strain DSM 6601, which is used in humans to treat gastrointestinal dysfunctions and diseases. Whilst agrobacterium can only infect and colonize plants, E. coli is a bacterium that populates the human and animal intestine. Agrobacterium with its pTi plasmid growths acts to the detriment of plants; E. coli has a positive impact on the host organism through different effects, such as the production of Vitamin K, the constant training of the mucosa immunological system and the production of secretory immunoglobulin A. Thus, agrobacterium and E. coli are two completely different organisms, and the removal or even desirability of removal of the plasmids would not be viewed by someone having ordinary skill in this art as being equivalent or even suggestive of each other. Briefly, the exchange of the sequence of the culture media is essential for the success described in our application.

Regarding the paper by Alexeyev et al., the Examiner is of course correct that this reference describes the introduction of a tetracycline-resistant cassette. However, this is not described for E. coli strain DSM 6601. Also, our particular combination of steps leading to a plasmid-free DSM 6601 strain, as set forth in claims 2-6, is not mentioned at all.

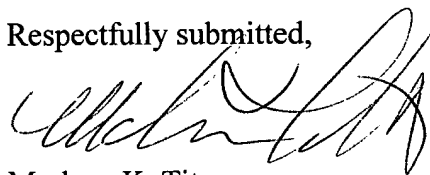
It is submitted that someone having ordinary skill in the art would not have found our method of claims 2-6 obvious with any or all of the four cited references in hand.

To assist the Examiner in understanding the difficulties that were necessary for the inventors to overcome in achieving our claimed invention, we provide here a copy of an article by Schultz et al. (2005), "Green fluorescent protein for detection of the probiotic microorganism Escherichia coli strain Nissle 1917 (EcN) in vivo", Journal of Microbiological Methods 61, 389-398. In this article, the original, plasmid-containing E. coli DSM 6601 strain is genetically manipulated to carry a foreign plasmid with the genetic information for the green fluorescent protein. As shown and concluded therein, the foreign plasmid was not stable in vivo (see Abstract and the discussion beginning on page 395). Manipulation of DSM 6601/Nissle 1917 to alter its plasmid content had limited success in Schultz et al. We submit this paper to show that, even as late as 2005, the construction of a plasmid-free DSM 6601 derivative is not a routine or obvious matter.

Reconsideration of this rejection is respectfully requested.

In summary, all of the Examiner's outstanding rejections and objections have been addressed, and the application is believed to be in allowable form. Notice to that effect is earnestly solicited. If the Examiner has any questions or would like to make suggestions as to claim language, she is encouraged to contact Marlana K. Titus at (301) 977-7227.

Respectfully submitted,



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Green fluorescent protein for detection of the probiotic microorganism *Escherichia coli* strain Nissle 1917 (EcN) in vivo

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Abstract

Probiotic microorganisms are defined as viable nutritional agents conferring benefit to the health of the human host. Especially, *Escherichia coli* strain Nissle 1917 (EcN) was shown to be equally effective as mesalazine in the maintenance of remission in ulcerative colitis (UC). Presumably, the therapeutic effect of EcN is linked to the presence of the strain in the region of interest; however, it remains difficult to follow the orally administered strain on its passage through the complex microbial environment of the intestine in vivo, inhabited dominantly by various *E. coli* strains, using traditional culturing methods. In this study we transformed EcN and a wild-type *E. coli* from a laboratory rat (EcR) with a plasmid carrying a *gfp* gene (pUC-*gfp*) to obtain EcN- and EcR-GFP to allow in vivo detection without alteration of strain-specific characteristics. Analysis of different strain-specific characteristics included the measurement of stimulation of IL-8 secretion and adhesion in vitro using the epithelial cell line HT-29. The kinetics of intestinal distribution in mice and colonization properties in rats following oral administration was studied in vivo. Detectability of the strain in histologic specimens was analysed using fluorescence microscopy and immunohistochemistry. The identity of fluorescent *E. coli* strains isolated from stool samples, Peyer's patches (PP) and mesenteric lymph nodes (MLN) was determined by REP-PCR. We were able to demonstrate that EcN and EcN-GFP do not differ in stimulation of IL-8 secretion or adhesion to HT-29 cells. In vivo, EcN-GFP colonies were readily detectable by fluorescence microscopy in luminal samples and also by immunohistochemistry in histological sections allowing analysis of the kinetics of the intestinal passage following oral administration. Translocation of fluorescent and non-fluorescent bacteria into PP and MLN was noted at 6 h post oral administration. EcN-GFP was detectable initially for 14 days in faecal samples of rats, while EcR-GFP was detectable throughout the whole experiment (45 days). Challenge with ampicillin at day 45 demonstrated continuing presence of EcN-GFP in small numbers by

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reappearing fluorescent colonies. The plasmid was not stable in vivo since non-fluorescent EcN colonies were detected also in faecal samples by REP-PCR.

In summary, transformation of EcN to obtain EcN-GFP in our study had no detectable influence on the probiotic microorganism regarding adhesion on and induction of IL-8 secretion of HT-29 cells and allows the detection in mixed microbial environments in vivo but the stability of EcN-GFP in vivo is limited.

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Keywords: Probiotics; Green fluorescent protein; *E. coli* Nissle 1917

1. Introduction

Probiotic microorganisms, such as different Lactobacilli, Bifidobacteria, and Enterobacteriaceae, are defined as viable nutritional agents conferring benefit to the health of the human host (Lilly and Stilwell, 1965). Clinically, beneficial effects of probiotics have been demonstrated for the treatment of infectious diarrhea in infants (Isolauri, 2003), amelioration of side-effects of antibiotic therapy (Cremonini et al., 2002), prevention of allergies (Bienenstock et al., 2002), and treatment of inflammatory bowel diseases (IBD) (Schultz et al., 2003a,b). Especially, *Escherichia coli* strain Nissle 1917 (EcN) was shown to be equally effective as mesalazine in the maintenance of remission in ulcerative colitis (UC) (Kruis et al., 1997, 2004; Rembacken et al., 1999).

The detailed mechanisms by which probiotic bacteria mediate their effects are beginning to be elucidated (Rachmilewitz et al., 2004) but are still not fully understood and seem complex and diverse among different probiotic preparations (Bai et al., 2004; Ulisse et al., 2001). Studies in humans, animals, and in vitro suggest that probiotic bacteria modulate the composition of the intestinal microflora (Tannock et al., 2000) or enhance the intestinal mucosal barrier (Mack et al., 1999; Madsen et al., 2001). A direct, anti-inflammatory influence on the intestinal immune system has also been suggested (Rachmilewitz et al., 2004; Malin et al., 1996; Schultz et al., 2003a,b).

Further in vitro experiments have suggested that probiotic bacteria are able to immunomodulate the cytokine response of intestinal mucosal cells in response to luminal antigens (Borruel et al., 2003), but in vivo confirmation with regard to the complex relationships involving possible interspecies and intraspecies interactions with orally administered

probiotic bacteria are rare (Boudeau et al., 2003; Mack et al., 1999).

Presumably, the therapeutic effect of a probiotic organism can be linked to its presence in the region of interest. Especially with the probiotic EcN it remains difficult to follow the orally administered strain on its passage through the complex microbial environment of the intestine in vivo, inhabited dominantly by different *E. coli* strains, using traditional culturing methods (Simon and Gorbach, 1984).

For the present report, we transformed EcN with a high-copy number plasmid carrying a modified *gfp* gene to obtain EcN-GFP, suitable for in vivo detection in mixed cultures or in histological sections. We further analysed the impact of labelling on strain-specific properties in vivo and in vitro and addressed questions regarding colonization kinetics and host cell interaction.

2. Methods and materials

2.1. Experiments in vitro

2.1.1. Bacterial strains and transformation

EcN was obtained from Ardeypharm GmbH, Herdecke, Germany. A wild-type strain of *E. coli* (EcR) was cultured from the faeces of a laboratory rat. The isolates were maintained on Columbia blood agar (CBA) and MacConkey agar plates (Merck, Darmstadt, Germany). Transformation of EcN and EcR to obtain EcN-GFP and EcR-GFP with plasmid pUC-*gfp* was performed using standard techniques (Sambrook et al., 1989). In brief, pUC-*gfp* was derived from the high-copy number vector pUC18 by insertion of a modified *gfp* gene under control of the *lac* repressor, allowing single cell detection by fluorescence microscopy (Crameri et al., 1996). Bacterial cells harbouring the plasmid were maintained on Mueller–Hinton-agar (Merck) or in Luria–Bertani broth (LB; Merck) in the presence of 100 mg/l ampicillin (Ratiopharm, Ulm, Germany). Fluorescence was

detected using an UV lamp (Micro-Bio-Tec, Gießen, Germany; 365 nm, 2×4 W) or a fluorescence microscope (Leica DMR X, Leitz, Wetzlar, Germany; fluorescence lamp Osram HBO 100 W, excitation filter 450–490 nm, band-elimination filter 515–560 nm). EcN-GFP was cured from the plasmid (EcN-cGFP) by repeated subculture to antibiotic-free media until disappearance of fluorescence and loss of resistance to ampicillin.

2.1.2. Stimulation of HT-29 cells and determination of interleukin (IL)-8 secretion

HT-29 intestinal epithelial cells at a concentration of 200,000/well of a 24 well plate were incubated at 37 °C in room air supplemented with 10% CO₂ for 24 h in antibiotic-free Dulbecco's MEM medium (GIBCO/BRL Invitrogen, Karlsruhe, Germany, 1 g/l D-glucose; 3.7 g/l NaHCO₃; 1.02 g/l N-acetyl-L-alanyl-L-glutamine) supplemented with 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany), non-essential amino acids, and 1% Na-Pyruvate (Biochrom, Berlin, Germany). Following renewal of the medium, co-incubation of EcN or EcN-GFP at concentrations from 1×10^2 – 1×10^{10} CFU/ml and HT-29 cells was performed for 16 h, and IL-8 was determined in the supernatant by ELISA (EH2-IL-8; Endogen, Perbio Science, Bonn, Germany). Viability of the cells was determined by trypan blue exclusion. As positive control, stimulation of the cells with 125 µg TNF-α/well (R and D Systems, Wiesbaden-Nordenstadt, Germany) was performed. All experiments were performed in duplicate and repeated three times.

2.1.3. Adhesion assay

To test for the influence of the labelling of EcN-GFP on adhesion to HT-29 intestinal epithelial cells, a standard adhesion assay was used (Boudeau et al., 2003). Bacteria (EcN, EcN-GFP, *E. coli* HB101 (as control)) were cultivated in liquid LB broth over night at 37 °C without agitation. Of a dilution ($A_{600}=0.01$) from the overnight culture, 8 µl were used to infect a confluent HT-29 monolayer in a well of a microtiter plate. Confluency of the HT-29 cell monolayer was reached after 24 h incubation under cell culture conditions. The infected HT-29 cells were incubated under cell culture conditions for 2 h. Each well was subsequently washed with 100 µl EBSS (Earl's balanced salt solution) three times. Addition of 100 µl Triton X-100 and shaking for 20 min resulted in lysis of the epithelial cells. Appropriate dilutions of the lysate were plated on LB agar plates. The number of HT-29 associated CFU was counted (Oelschlaeger and Tall, 1997).

All experiments were repeated at least three times and three wells per condition were used.

2.2. Experiments in vivo

2.2.1. Intestinal distribution and kinetics of EcN-GFP in mice following oral administration

Commercially available Balb/C mice at 8–12 weeks and 18–20 g body weight (Charles River, Sulzfeld, Germany) were orally inoculated with a single-dose of 200 µl sterile saline containing 5×10^{10} CFU/ml EcN-GFP. At 1, 3, 6, 12, 24, 48, and 72 h post gastric lavage, two animals and one control animal were sacrificed by CO₂ asphyxiation. Liver, spleen and all detectable MLN and PP were removed aseptically before the intestine was opened. Cardiac blood was taken and used undiluted. To minimize the risk of contamination, the intestine was opened at the end of the procedures to take samples from luminal contents from the stomach, cecum, and rectum. All samples were weighed, homogenized, serially diluted in saline, and cultured on CBA and MacConkey plates (Merck, Darmstadt) overnight at 37 °C in air supplemented with 5% CO₂. Numbers of CFU were normalized by weight.

2.2.2. Immunohistochemistry for EcN-GFP

Intestinal tissue was washed vigorously in sterile PBS, embedded (Tissue Tek® O.C.T. compound 4583; Zoeterwoude, The Netherlands), and frozen in liquid nitrogen using an iso-pentane heat buffer to prevent snap-freezing. Cryosections of intestinal tissue were first blocked with FCS and then incubated in PBS containing anti-GFP rabbit polyclonal IgG antibody. As secondary antibody, an anti-rabbit IgG-HRP was used (Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturer's instructions of the VECTOR®NovaRED substrate kit (Vector Laboratories, Burlingame, CA).

2.2.3. Colonization of rats and intermittent antibiotic challenge

Commercially available Fisher rats (Charles River) ($n=4$) were treated with ampicillin (50 mg/kg body weight) in drinking water for 3 days and then challenged on three consecutive days by gastric lavage with 1000 µl PBS containing $3\text{--}4 \times 10^9$ CFU of EcN-GFP or EcR-GFP. Stool samples (1 g wet weight) were collected on days 0, 3, 7, 10, 14, 17, 23, 30, 37, and 45, diluted and homogenized in 100 µl sterile saline. Serial dilutions were cultured on blood agar plates at aerobic conditions. After 24 h, plates were inspected under UV light. As soon as the absence of fluorescent colonies from faecal samples was noted (day 14 and day 37), rats were given ampicillin (50 mg/kg body weight) (days 15–17 and days 38–40) or clindamycin (50 mg/kg body weight; days 31–33) in drinking water, followed by culture of the faecal flora.

2.2.4. Typing of *E. coli* isolates by repetitive extragenic palindromic PCR (REP-PCR)

REP-PCR was performed according to Versalovic et al. (1991). Briefly, after adjustment of the concentration of genomic DNA, PCR was performed using primers REP-1 5'-GCGCCGICATCAGGC-3' and REP-2 5'-ACGTCT-TATCAGGCCTAC-3' with 30 cycles of 94 °C for 1 min, 40 °C for 1 min and 65 °C for 8 min. Patterns with different bands were considered to represent different *E. coli* strains.

2.2.5. Detection of EcN-GFP by fluorescence microscopy

Tissue sections from the stomach, jejunum, ileum, caecum, colon, and rectum were embedded (Tissue Tek®) and frozen in liquid nitrogen using an *iso*-pentane heat buffer to prevent snap-freezing and tissue damage. Frozen samples were stored at -70 °C until further use. Frozen sections (3–4 µm thick) and H+E stained sections were examined using native contrast and fluorescent light. Images were taken with a digital camera (RT Color Spot 2.2.1., Diagnostic Instruments Inc., St. Sterling Heights, MI).

2.3. Statistical analysis

For statistical analysis, Sigma Stat 2.03 (SPSS Inc., Chicago, IL 60606 USA) software was used. To control for normally distributed variables, the Kolmogorow–Smirnow

test was used, followed by a *t*-test. A $p < 0.05$ was regarded as significant, while higher values were regarded as non-significant (ns).

3. Results

3.1. Experiments in vitro

3.1.1. Stability of EcN-GFP in vitro

Transformation of EcR and EcN with plasmid pUC-*gfp* resulted in clones EcR-GFP and EcN-GFP with uniform and bright display of fluorescence of single colonies after 24 h on LB agar plates containing 100 mg/l ampicillin. Daily serial subculture of EcN-GFP on Mueller–Hinton-agar without ampicillin demonstrated that after 14 days more than 50% of approximately 1000 CFUs harboured the plasmid, as evidenced from fluorescence. Subcultures of non-fluorescent colonies failed to grow on Mueller–Hinton-agar supplemented with ampicillin. Under the microscope at $\times 1000$ magnification and fluorescent light, single fluorescent bacterial cells with different intensity of fluorescence could be detected (data not shown).

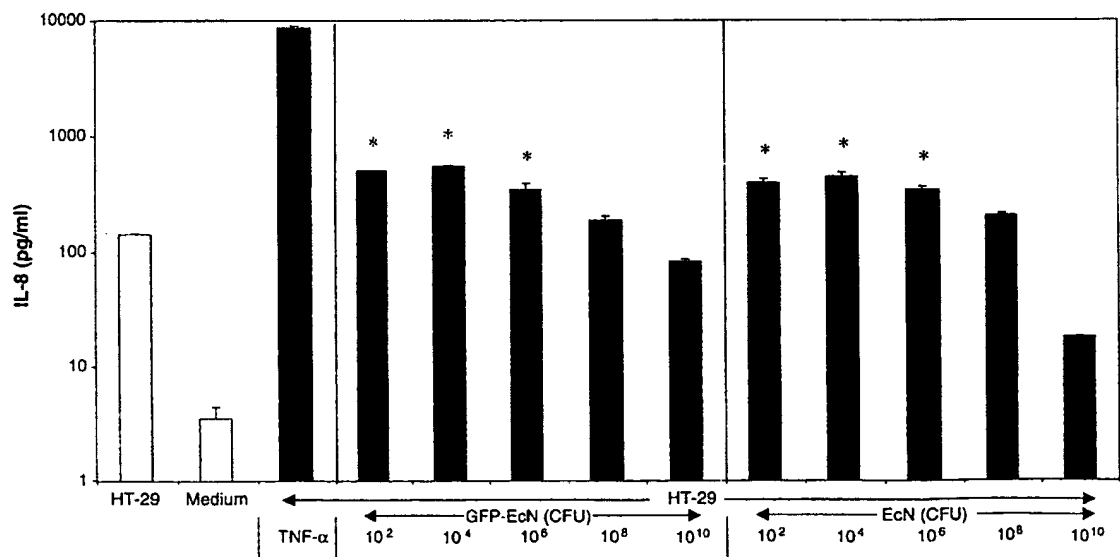


Fig. 1. Comparison of the ability of EcN and EcN-GFP to stimulate the secretion of IL-8 in HT-29 epithelial cells. There was no significant difference between the original EcN and EcN-GFP. * $p \leq 0.05$ vs. unstimulated HT-29 cells. Shown is one of at least three experiments.

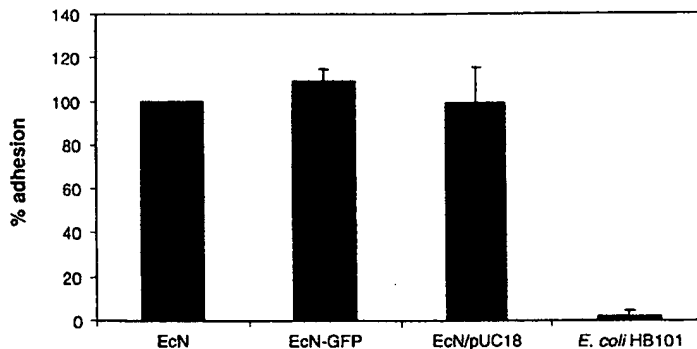


Fig. 2. Adhesion of different bacterial strains to HT-29 epithelial cells. No difference in adherence efficiency was observed with EcN-GFP and EcN containing the empty plasmid vector compared with EcN. *E. coli* HB 101, with no adhesion to epithelial cells, was used as negative control.

3.1.2. In vitro stimulation of IL-8 secretion by HT-29 cells

Secretion of IL-8 from HT-29 cells was stimulated by co-incubation with EcN and EcN-GFP in vitro. IL-8 secretion was dose-dependent with maximum values observed at concentrations of 10^2 to 10^4 CFU/ml. No significant difference in secretion of IL-8 could be demonstrated for stimulation with EcN or EcN-GFP (Fig. 1).

3.1.3. In vitro adherence to HT-29 cells

Adherence to host epithelial tissue was investigated in vitro by co-incubating EcN and EcN-GFP with HT-29 cells for 1–3 h. No alteration of in vitro adhesion of

EcN-GFP in comparison to EcN to HT-29 cells was noted (Fig. 2).

3.2. Experiments in vivo

3.2.1. Intestinal passage and localisation of EcN-GFP in vivo

We analysed the kinetics of the intestinal passage of EcN-GFP following oral administration in more detail in Balb/c mice. After a single dose of EcN-GFP, fluorescent colonies were detectable in subcultures of faecal samples on CBA plates (Fig. 3). One hour after ingestion, EcN-GFP was detectable in high numbers in the stomach and in lower numbers in the caecum, but

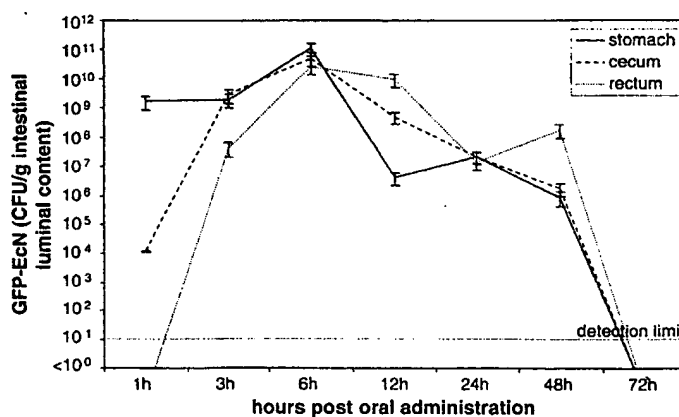


Fig. 3. Intestinal passage of EcN-GFP following single oral administration. EcN-GFP was readily detectable in intestinal luminal content, cultured on CBA up to 48 h.

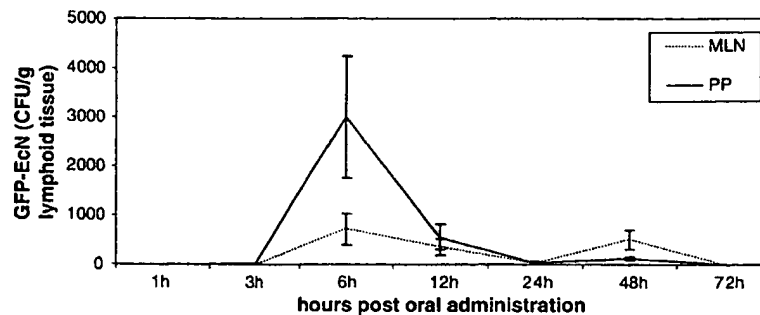


Fig. 4. Bacterial translocation of EcN-GFP to mesenteric lymph nodes (MLN) and Peyer's patches (PP). Detection of fluorescent colonies following homogenization of lymphoid tissue and subculturing on CBA. No fluorescent colonies were detected in blood or splenic tissue.

not in the rectum. At 6 h post administration the microorganism was equally present throughout the intestine (stomach, cecum, and rectum) with maximum concentrations of approximately 5×10^{10} CFU/g faeces in all intestinal sections as determined by subculture. At this time-point, viable microorganisms (e.g. *enterococci*, *E. coli* with and without fluorescence) were subcultured for the first time from at least three homogenized PP and MLN from different locations of the intestine (Fig. 4). No organisms were detected in the spleen, liver or blood of the animals at all time-points. By fluorescent microscopy and immunohistochemistry, it became apparent that the organisms were mainly localized close to the epithelial surface of the stomach, cecum, and rectum and also in the lumen of the intestine (Figs. 5 and 6).

3.2.2. Colonization of rats and intermittent antibiotic challenge

The colonization of Fisher rats by EcN in comparison to EcR was monitored after a 3-day oral antibiotic pre-treatment with ampicillin followed by a 3-day period of oral administration of EcN-GFP or EcR-GFP. Following oral administration of $3\text{--}4 \times 10^9$ CFU the numbers of EcN-GFP in faeces steadily declined, until at day 14 faecal cultures for EcN-GFP were negative. In contrast, the numbers of EcR-GFP remained stable. Following renewed administration of ampicillin on days 15–17, concentrations of EcN-GFP and also EcR-GFP increased to reach a maximum of 1×10^{11} CFU/g on day 17. Again, numbers of EcN-GFP declined with time and EcN-GFP was not detectable at day 45, despite administration of

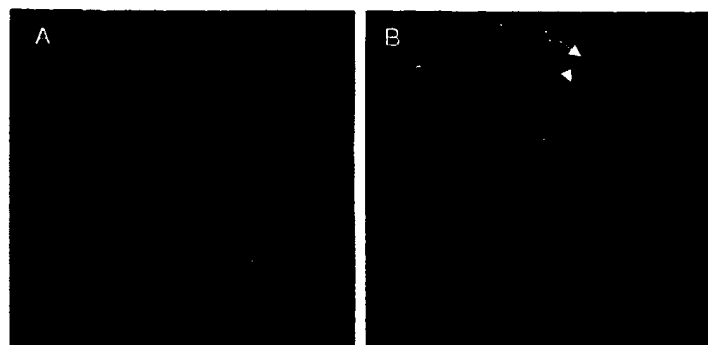


Fig. 5. Fluorescence microscopy of histological sections of the caecum from untreated controls (A, autofluorescence of intestinal tissues) and from mice challenged with EcN-GFP (B). EcN-GFP appears as a bright band close to the epithelial surface on the bottom of the crypt (arrows) ($\times 400$).

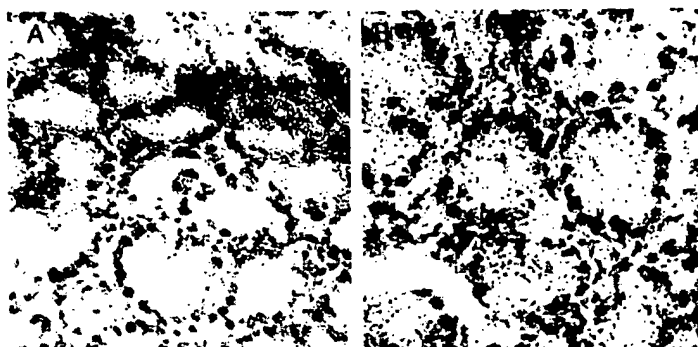


Fig. 6. Immunohistochemical analysis of the caecum of an untreated mouse (A) and an animal, challenged with EcN-GFP (B). EcN-GFP appears red-brown on the luminal surface of the epithelium. The section is counterstained with H and E ($\times 400$).

clindamycin at days 31–33 or ampicillin at days 38–40. EcR-GFP concentrations remained stable up to day 45 (Fig. 7).

3.2.3. Stability of *pUC-gfp* as a marker in vivo

REP-PCR of EcN-GFP revealed a pattern distinct from patterns from non-fluorescent *E. coli* cultured from the faeces of rats prior to feeding (data not shown). After feeding, all colonies with fluorescence demonstrated the pattern of EcN-GFP, however, non-fluorescent colonies with the pattern of EcN were found (data not shown).

4. Discussion

Probiotic microorganisms are defined as viable nutritional agents conferring benefits to the health of the human host (Lilly and Stilwell, 1965). Despite mounting evidence for clinical benefits of EcN (Schultz et al., 2003a,b), the mechanisms by which this organism mediates its anti-inflammatory effects are just beginning to be elucidated but still are not well understood (Rachmilewitz et al., 2004). In the case of EcN, this is largely due to technical difficulties since it remains a challenge to study the fate of this microorganism, administered orally, in a complex microbial environment in vivo such as the human intestinal bowel flora, in which numerous *E. coli* strains represent the dominant enterobacterial species (Adlerberth et al., 1998; Kühn et al., 1986; Nowrouzian et al., 2003). Furthermore, it has been shown that the faecal

flora does not represent the whole diversity of the intestinal microbial environment. Especially in IBD, a different composition of the microflora originated from healthy and diseased sections of the gut were seen close to or within the mucosal layer (Swidsinski et al., 2002; Seksik et al., 2003).

Labelling of EcN with GFP enabled us to follow the intestinal passage of the orally administered strain EcN-GFP in mice to document bacterial translocation in PP and MLN and to compare colonization properties of EcN with that of an *E. coli* strain isolated from rat faeces, in-vivo.

For that purpose, *gfp* under the control of the *lac* promoter was subcloned into the high-copy plasmid pUC18 to obtain high-level expression of GFP, necessary for single-cell detection at higher magnification in mixed cultures. However, since expression of GFP to high intracellular concentrations may influence main functions of the host strain, we compared EcN-GFP with the unlabelled strain in in vitro assays. EcN is known to stimulate NF- κ B-dependent IL-8 secretion in HT-29 cells (Lammers et al., 2002). In our experiments, plasmid-carriage by EcN did not influence the dose-dependent stimulation of IL-8 secretion in HT-29 cells. Adhesion of EcN and EcN-GFP to HT-29 cells was studied, using a standard adhesion assay. Labelling of EcN with GFP did not alter the adhesion properties in vitro to HT-29 cells.

To link the effect of a probiotic organism to its presence in the region of interest, we analysed the use of EcN-GFP on a cellular level in a mouse model. Following oral administration of a single dose without

prior antibiotic treatment, the passage of EcN-GFP was monitored by fluorescent microscopy and immunohistochemistry of histological sections and by culture of luminal content on CBA. Due to the high expression of GFP, EcN-GFP was readily detectable close to the intestinal epithelial cell layer by fluorescence microscopy (Fig. 6) as well as in luminal contents by subculturing on CBA. Immunohistochemical analysis confirmed the location of EcN-GFP (Fig. 6). Bacterial translocation from the gastrointestinal tract of mice into organs of the intestinal immune system occurs on a regular basis (Berg, 1983; Wells et al., 1987). However, for the first time, translocation into PP and MLN of an orally administered viable probiotic microorganism was documented. This finding opens the possibility that probiotic microorganisms mediate their immunomodulatory effects through a direct contact with immunocompetent cells of the intestinal immune system (Rachmilewitz et al., 2004; Schultz et al., 2003a,b).

Plasmidic carriage of a marker gene may likely cause its loss at growth conditions without selective

pressure. We demonstrated that pUC-*gfp* was detectable in clones of EcN after subculturing at non-selective conditions in vitro after 14 days. At the same time, as expected, clones with concomitant loss of fluorescence and resistance to ampicillin were observed, suggesting the loss of the plasmid. To apply the transformed EcN in vivo and study stability and detectability of the plasmid as well as colonization in comparison to an *E. coli* strain, isolated from rat faeces, we investigated the modified strain in a rat model. While EcN-GFP was detectable for up to 14 days following administration, the rat-derived EcR remained at a stable concentration throughout the whole experiment (day 45), most likely due to better adaptation and adhesion mechanisms. After direct selection for EcN-GFP by the administration of ampicillin, EcN-GFP was again detectable and re-grew to high numbers in the faeces, but numbers again declined until day 45. Re-growth of EcN-GFP was not recorded after the administration of clindamycin. The true number of EcN-GFP in faeces is probably underestimated due to the loss of

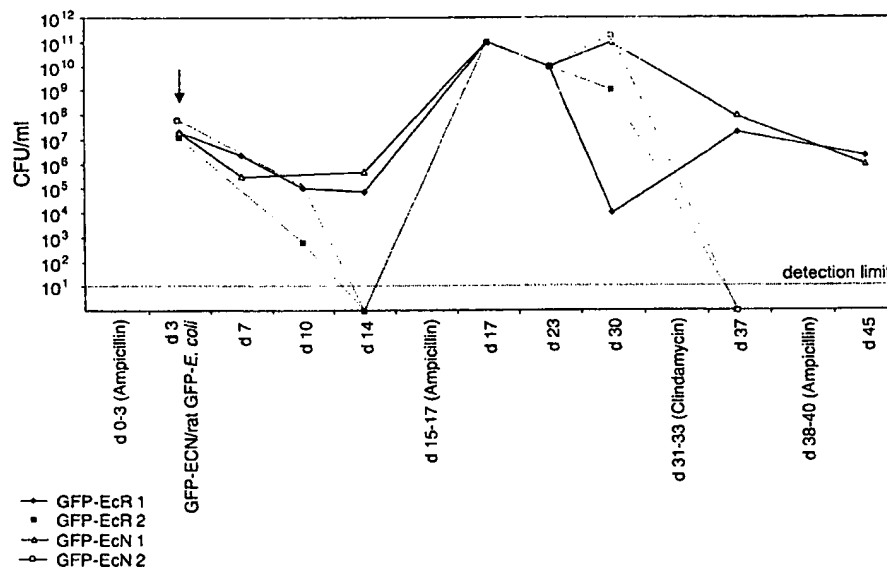


Fig. 7. Concentration of fluorescent colonies in faecal samples of rats, following a single oral challenge with a specific rat-derived *E. coli* (EcR-GFP ■/◆) and EcN-GFP (□/△) to study the colonization properties and the influence of antibiotics on the colonization process. A single dose of EcN-GFP and EcR-GFP were administered orally following three days of oral ampicillin. EcN-GFP was initially detectable in luminal samples for 14 days and again after oral antibiotic treatment with ampicillin up to day 37. The concentration of the rat-derived EcR was stable throughout the experiment until day 45. Administration of clindamycin on days 31–33 did not influence the luminal concentration of either EcN-GFP or EcR-GFP.

plasmid as demonstrated by REP-PCR. Faeces might also not truly represent the bacterial spectrum at different locations of the gut. Apart from GFP expression in vivo being not stable due to the lack of selection pressure and loss of plasmid, the possibility remained that the plasmid was acquired by other strains of the resident bowel flora. However, as documented by REP-PCR analysis from individual *E. coli* clones from faeces, all fluorescent and also numerous non-fluorescent colonies exhibited patterns undistinguishable from EcN. This indicates that pUC-*gfp* was not acquired by other enterobacteriaceae. However, anaerobic bacteria were not analysed.

In summary, GFP expressed by the probiotic *E. coli* strain Nissle 1917 from a high-copy plasmid allowed for the first time a study on the intestinal passage of an orally administered probiotic microorganism. In agreement with our in vitro observations, the strain adheres to the intestinal epithelial layer and immunomodulatory effects might be mediated by direct contact with immunocompetent cells of the host following translocation of viable microorganisms into PP and MLN. A limitation to the use of EcN-GFP is the instability of the marker in vivo due to the lack of applied selection pressure. This leads to underestimation of the concentration in faecal subcultures. Acquisition of the plasmid by other microorganisms was not observed. However, the use of EcN-GFP opens an array of experimental possibilities like the study of microbial–host interactions on the cellular level, the influence of the resident microflora, and concurrent antimicrobial treatment on colonization.

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